EFFECT OF DIETHYLPYROCARBONATE ON pH-DRIVEN MONOAMINE UPTAKE BY CHROMAFFIN GRANULE GHOSTS

M. F. ISAMBERT and J. P. HENRY

Service de Biochimie-Physique, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

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1. Introduction

Chromaffin granules, the catecholamine storage organelles of adrenal medulla, accumulate monoamines by an active process which involves 2 different entities: an electrogenic ATP-dependent H^+-translocase which generates a proton electrochemical gradient $\Delta\mu_{H^+}$ (interior acid and positive) [1–3] and a specific monoamine carrier which mediates the coupling between $\Delta\mu_{H^+}$ and substrate accumulation [4–6]. Kinetics and pharmacological properties of the latter have been described [7,8], but little is known about its biochemistry. Diethylpyrocarbonate, a histidine specific reagent, has been shown to affect lactose/H^+ symport in *Escherichia coli* membranes by directly reacting with the *lac* carrier [9]. A similar reaction was reported for proline and D-lactate transport [9]. We investigated the possibility that DEPC might also react with the monoamine carrier of chromaffin granule membranes. Monoamine/proton antiport was assayed independently of the H^+-translocase by imposing a pH-gradient (interior acid) on chromaffin granule ghosts [5,8,10].

2. Materials and methods

2.1. *Materials*

DEPC was purchased from Sigma. Stock solutions (120 mM) were prepared in absolute ethanol and were used immediately. Nigericin and OX-V were gifts of Dr A. Stempel (Hoffman-La Roche) and Dr B. S. Cooperman, respectively. [^{3}H]Dihydrotetrabenazine (12 Ci/mmol) was prepared as in [11]. 5-Hydroxy [G-^{3}H]tryptamine creatinine sulfate (18 Ci/mmol), 1-[7,8-^{3}H]Noradrenaline (12 Ci/mmol), [7-^{14}C]tyramine hydrochloride (50 mCi/mmol) were from Amersham, [^{14}C]methylamine hydrochloride (38 mCi/mmol) was from CEA.

Abbreviations: DEPC, diethylpyrocarbonate; OX-V, bis (3-phenyl-5-oxoisoxazol-4-yl) pentamethine oxonol; Hepes, *N*-2-hydroxyethyl piperazine–*N'*-2 ethane sulfonic acid; Mes, 2-(*N*-morpholino) ethanesulfonic acid; 5-HT, 5-hydroxytryptamine

2.2. *Chromaffin granule ghosts*

Ghosts were derived from purified chromaffin granules by hypoosmotic lysis [12], frozen in liquid nitrogen and stored at −80°C.

2.3. *DEPC-treatment of membranes*

Membranes were thawed diluted by 3 vol. water, centrifuged for 15 min at full speed in an Airfuge centrifuge (Beckman) and resuspended at 4–5 mg protein/ml in 10 mM potassium phosphate buffer (pH 6.0) containing 0.15 M KCl (buffer A). DEPC (generally 1.2 mM, final conc.) or absolute ethanol (1%, final conc.) were added under stirring and the mixture was kept at room temperature for 15 min. When nigericin and K^+ were used to generate the pH-gradient [13] the mixture was diluted ~20-fold in 10 mM Tris–Hepes buffer (pH 7.5) containing 0.15 M KCl (buffer B), centrifuged at 160 000 × g_{av} for 20 min and resuspended at 10–20 mg protein/ml in the same buffer. When nigericin was not used, samples were washed in buffer A or, where indicated, in a similar buffer in which K^+ was substituted for by Na^+.

2.4. *ΔpH-driven monoamine uptake*

Nigericin was routinely used to generate pH-gradient [13]. Membranes resealed in buffer B (5 μl aliquots containing 0.05–0.1 mg protein) were diluted 100-fold

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in 10 mM Tris–Hepes (pH 8.5) containing 0.3 M sucrose, 2 mM $MgSO_4$, 10 μM nigericin and 1 μM [^{3}H]noradrenaline or 5-[^{3}H]HT (~500 000 cpm). Controls contained in addition 20 μM tetrabenazine. Samples were incubated at 37°C, diluted by 1 ml ice-cold 0.3 M sucrose, 10 mM Tris–Hepes buffer (pH 8.5) and filtered on HAWP Millipore filters. Filters were washed twice with 2 ml of the same buffer and their radioactivity measured in 5 ml either toluene–PPO–POPOP (after drying) or Aqualuma (without drying). Results were corrected for non-carrier mediated amine retention by subtracting control values measured in presence of tetrabenazine.

2.5. *Analytical techniques*

ATPase activity was measured as in [12]. Potential measurements with the probe OX-V were performed as in [14]. [^{3}H]Dihydrotetrabenazine binding was estimated by the centrifugation technique [11]. Proteins were assayed by the Lowry procedure.

3. Results

3.1. *Effect of DEPC treatment on ΔpH-driven monoamine uptake*

DEPC-treatment inhibited both initial rate and plateau level of monoamine uptake driven by a pH-gradient (inside acidic) (fig.1). The inhibition was often visible after a 10 s lag (see fig.2A, inset). With both noradrenaline and 5-HT as the transported amine, inhibition of the rate of uptake was repeatedly in the 30–70% range under various experimental conditions. Maximal effect required ~1.2 mM DEPC (fig.2B). The figure also shows the effect of DEPC on ghost ATPase and on the ATP-induced fluorescence changes of the extrinsic probe OX-V, which monitored transmembrane potentials generation by the ATP-driven H^+-translocase [14]. Both activities were inhibited by the reagent in the same concentration range as monoamine uptake.

The inactivation of 5-HT uptake by 1.2 mM DEPC was relatively independent of pH over 6.0–8.5, increasing from 50% at pH 6.0 to 70% at pH 7.5 (not shown). The reaction was routinely performed at pH 6.0 since *N*-ethoxyformyl derivatives are more stable at acidic pH [15]. Under these conditions, the inhibition was slow (fig.2A).

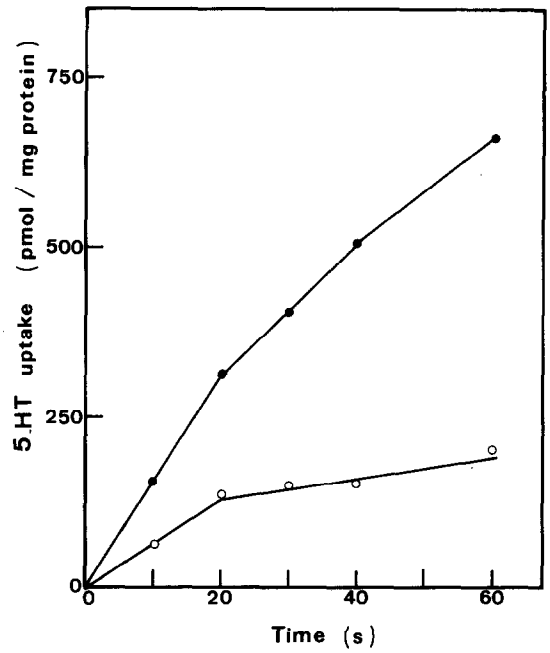

Fig.1. ΔpH-driven uptake of 5-HT by DEPC-treated membranes. Membranes were treated by 2 mM DEPC (○) or by 1% ethanol (●). 5-HT uptake was measured in presence of nigericin (see section 2.4).

3.2. *Effect of DEPC on passive properties of chromaffin granule membrane*

Since DEPC certainly modified various components of the membrane (see fig.2B), it was of interest to test if monoamine uptake inhibition did not originate in a non-specific change of the membrane properties. Methylamine is accumulated by ghosts which have an internal space acidic with respect to the medium [16]. This transport is not carrier-mediated [8] and it can be utilized to measure transmembrane pH-gradients [1]. Methylamine uptake was not affected in DEPC-treated vesicles (fig.3), indicating that modified membranes monitored pH-gradients similar to controls. The effect of DEPC on ΔpH-driven tyramine uptake was next investigated. Tyramine is not transported by the tetrabenazine-sensitive monoamine carrier when ghosts are energized by an imposed pH gradient [8] and this amine can thus be considered as a pH-probe. ΔpH-driven tyramine uptake by DEPC-treated membranes was resistant to 10 μM tetrabenazine and was 85% of control values with untreated vesicles, providing strong evidence for the argument that passive properties of the membrane were not modified.

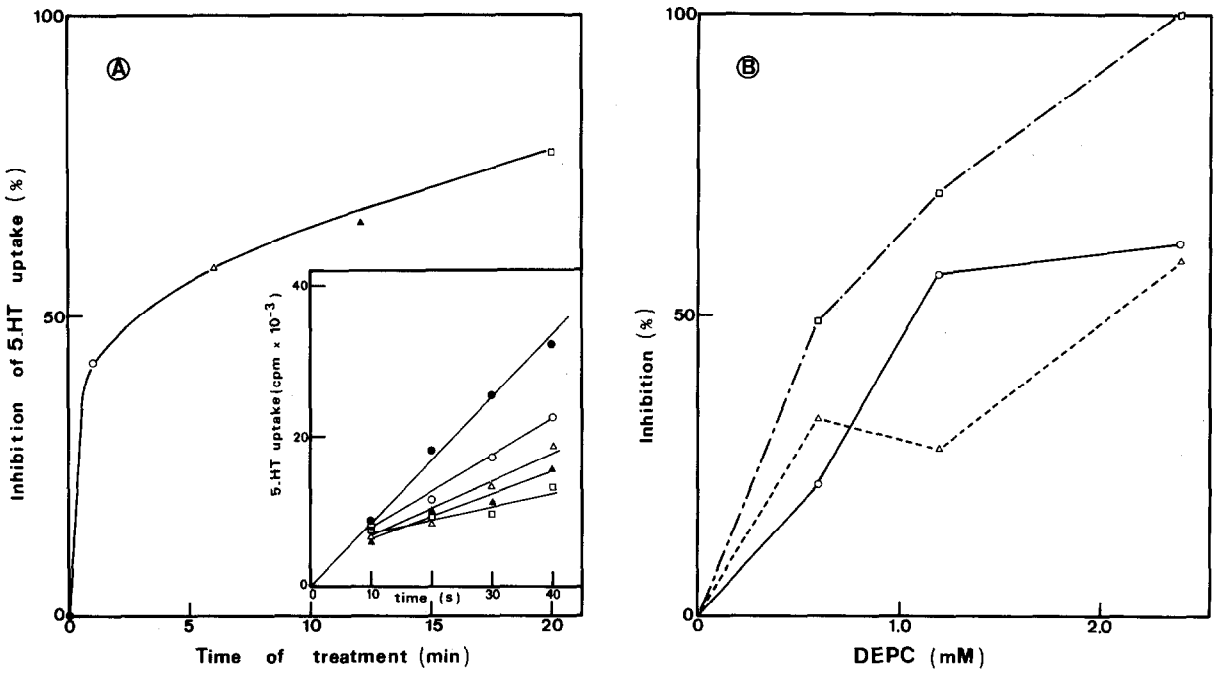

Fig.2. Time-course and dose-dependency of DEPC-modification. (A) Membranes were incubated with 1.2 mM DEPC at room temperature for the indicated period of time. They were washed and assayed for 5-HT uptake in presence of nigericin. Results are expressed as inhibition of the rate of uptake. Inset: Time-course of 5-HT uptake by modified membranes. (B) Membranes were treated with the indicated concentration of DEPC for 15 min at room temperature. 5-HT uptake (○), ATPase activity (△), and ATP-induced OX-V fluorescence change (□) inhibitions were measured in the same samples.

The permeability to K^+ of the membrane was investigated (fig.4), using the fluorescence of OX-V to monitor transmembrane potential [14]. Addition of K_2SO_4 (50 mM) did not polarize positively control or DEPC-treated ghosts resealed in a Na^+-containing medium. Such an effect was observed after addition of the K^+-ionophore valinomycin and was similar with both preparations. The lack of polarization in absence of the ionophore showed that DEPC did not increase the K^+ permeability of the membrane.

The absence of changes in the passive properties of the membrane suggested that DEPC altered the monoamine carrier. We were nevertheless unable to demonstrate a change of reactivity to DEPC associated

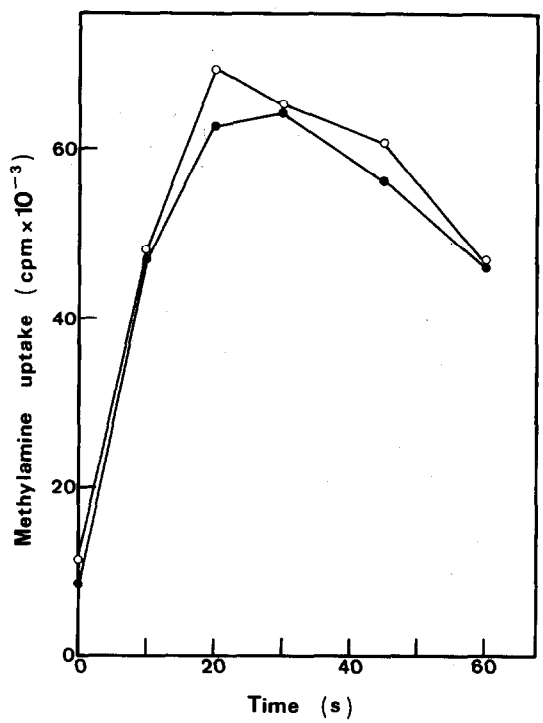

Fig.3. Methylamine uptake by DEPC-treated membranes. Control (●) and treated membranes (○) were resuspended in buffer A. Aliquots (10 μl) were diluted 20-fold in 10 mM Tris–Hepes (pH 9.2) containing 0.3 M sucrose and [^{14}C]-methylamine (0.16 μCi); final pH was 8.7. After incubation at 22°C, samples were processed as described for monoamine uptake, the radioactivity of the filters being measured without drying in 5 ml Aqualuma.

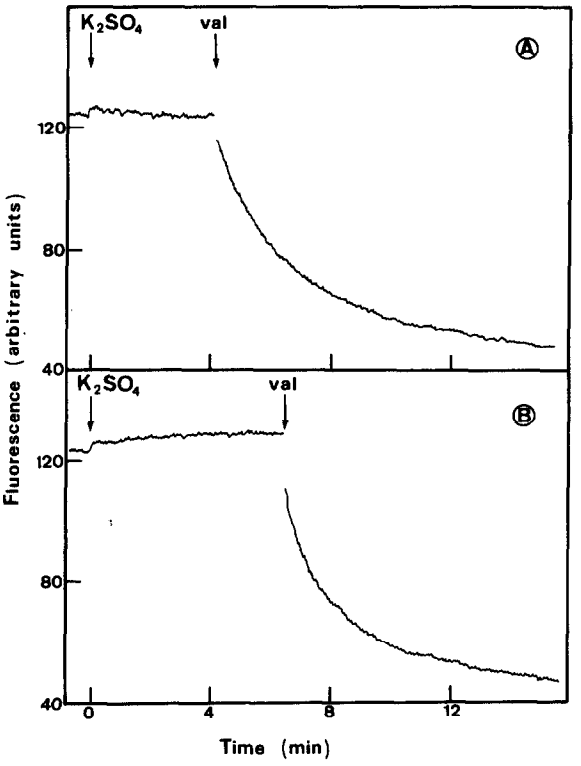

Fig.4. Effect of DEPC-treatment on K^+-induced OX-V effect. Membranes were treated for 15 min with 1% ethanol (A) or with 1.2 mM DEPC (B), as in section 2.3 but with Na^+ substituted for K^+ in buffer A. The cuvette contained in 2 ml final volume: 140 μg protein of control (A) or DEPC-treated (B) membranes resealed in Na^+-containing buffer, 1 μM OX-V, 0.3 M sucrose and 20 mM Mes–NaOH buffer (pH 6.6). After 3 min at 22°C, 500 mM K_2SO_4 (200 μl) was added, followed by 4 mM valinomycin (5 μl). Fluorescence (exc. 580 nm, em. 650 nm) was measured with a JY 3 C Jobin-Yvon fluorimeter. Relative fluorescence changes after valinomycin addition were 59.8% and 62.5% for control and DEPC-treated membranes respectively.

with protection or energization of the monoamine transporter. Noradrenaline (0.5 mM) did not protect membranes from inactivation by treatment at pH 7.6 with 1.2 mM DEPC. Energization of the vesicles by addition of 0.5 or 1 mM ATP prior to the addition of 2 mM DEPC did not change 5-HT uptake inhibition.

3.3. *Effect of imposed transmembrane potentials ΔΨ on ΔpH-driven monoamine uptake by DEPC-treated membranes*

We [6] and others [17,18] have shown that ΔΨ superimposed on pH-gradients changed noradrenaline rate of uptake. When the vesicle interior was polarized positively by treatment of the ghost membrane with valinomycin and addition of K_2SO_4 to the medium, the rate of uptake was increased. This experiment was repeated with DEPC-treated preparations. A positive polarization enhanced the rate of uptake of 5-HT (fig.5A) or noradrenaline (not shown) by modified vesicles. The stimulation factor was about the same or slightly higher with DEPC-treated than with controls (1.5–4-fold stimulation). Consequently the rate of uptake measured in presence of both a ΔpH and a positive ΔΨ was also inhibited by DEPC-treatment. We had shown that ghosts negatively polarized by a suitable K^+ gradient took up noradrenaline at a decreased rate [6]. This property was also shared by DEPC-treated vesicles (fig.5B).

3.4. *Kinetics of noradrenaline uptake in DEPC-treated vesicles*

Kinetics of ΔpH-driven noradrenaline uptake by DEPC-treated or control vesicles have been examined (table 1). The pH gradient was generated either by resuspension at pH 8.5 of ghosts resealed at pH 6.0 (exp. 1) or by nigericin in presence of external K^+ ions (exp. 2). Under the two sets of conditions, V_{max} was decreased by DEPC-treatment whereas K_m was not significantly altered.

3.5. *Binding of [^{3}H]dihydrotetrabenazine*

[^{3}H]Dihydrotetrabenazine, a derivative of the potent uptake inhibitor tetrabenazine, binds to saturable sites on chromaffin granule membranes [11]. Treatment of the membranes with DEPC changed neither the equilibrium dissociation constant K_d nor the number of binding sites (table 1).

4. Discussion

DEPC-treatment has been shown to inhibit ΔpH-driven monoamine uptake. This effect is not due to an inhibition of the ATP-dependent H^+-translocase since the H^+-pump was by-passed under the described conditions of uptake. It is not an artefact of the uptake assay since:

(i) Membranes were washed before being assayed;
(ii) Similar inhibitions were observed when pH-gradients were generated either by resealing ghosts at acidic pH or by a K^+-gradient in presence of nigericin.

A more rapid dissipation of the pH-gradient in DEPC-

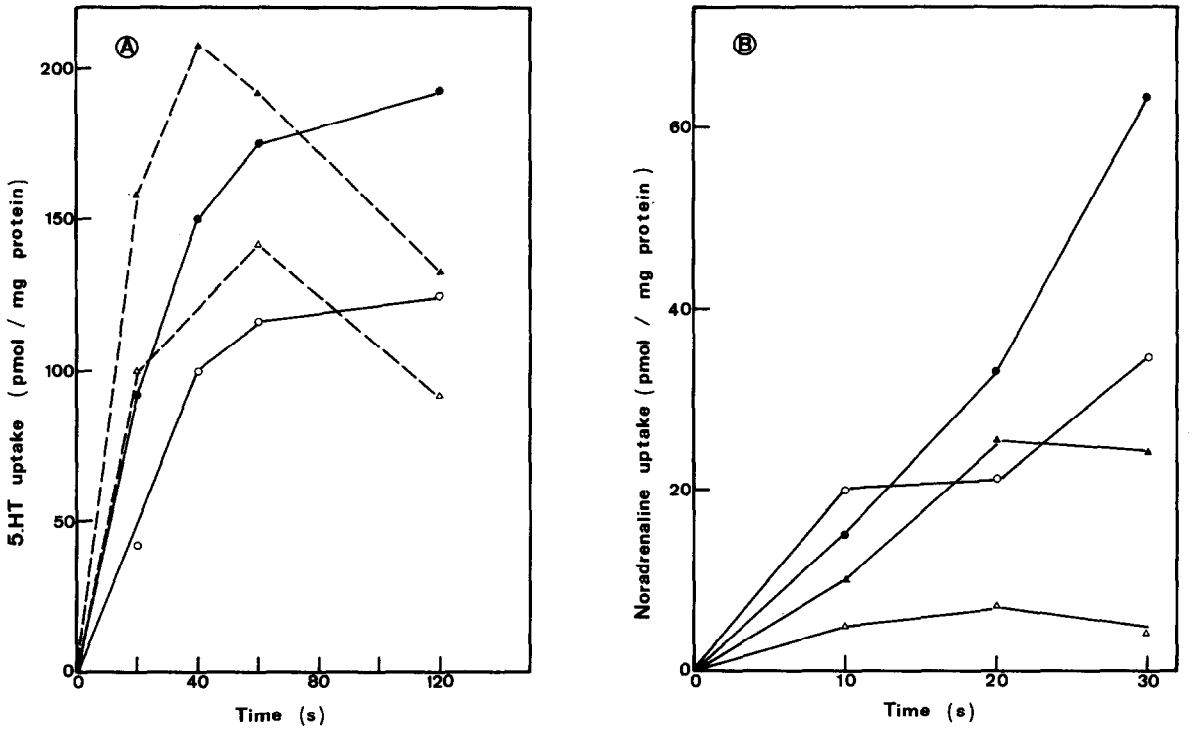

Fig.5. Effect of transmembrane potentials on ΔpH-driven monoamine uptake. (A) Effect of a positive $\Delta\Psi$. Membranes were treated as in section 2 but with Na^+ substituted for K^+ in buffer A and were washed in the same buffer. DEPC-treated (○,△) and control (●,▲) membranes were assayed in presence (▲,△) or in absence (●,○) of a positive $\Delta\Psi$ induced by valinomycin. The incubation mixture (200 μl) contained: 70 μg protein, 1 μM 5-HT (300 000 cpm), 0.3 M sucrose, 100 mM Hepes–KOH (pH 8.5) and, where indicated (▲,△) 5 μM valinomycin. (B) Effect of a negative $\Delta\Psi$. Membranes were treated and washed in buffer A (K^+ medium). Symbols are as in (A). The incubation mixture contained in 500 μl: 130 μg protein, 1 μM noradrenaline (× 10^3 cpm), 2 mM $MgSO_4$, 0.3 M sucrose, 10 mM Tris–Hepes buffer to give a final pH of 8.5 and, where indicated (▲,△) 5 μM valinomycin.

Table 1
Effect of DEPC-treatment on noradrenaline uptake kinetics and [^{3}H]dihydrotetrabenazine binding

	Control	DEPC-treated
Uptake of noradrenaline		
Expt 1[a]		
K_m (μM)	25	20
V_{max} (pmol . min^{-1} . mg $protein^{-1}$)	4000	2400
Expt 2[b]		
K_m (μM)	9.1	10.6
V_{max} (pmol . min^{-1} . mg $protein^{-1}$)	2630	920
Binding of dihydrotetrabenazine[c]		
K_d (μM)	0.07	0.062
B_{max} (pmol/mg)	155	153

[a] Expt 1 was performed as in fig.5A without valinomycin addition, but with noradrenaline as the substrate
[b] Expt 2: noradrenaline uptake was measured in presence of nigericin
[c] Tetrabenazine binding was measured on membranes treated in buffer A and washed by buffer B

treated vesicles than in controls can be ruled out since methylamine and tyramine uptakes, which depend only on the transmembrane pH-difference [8], were similar for both preparations. Acceleration of catecholamine leakage in modified vesicles is unlikely since initial rates of uptake were considered and since tyramine accumulation was unaffected. Indirect electrogenic effects, e.g., negative polarization of the DEPC-treated ghosts, is not a possible explanation in all experimental situations since inhibition was observed in sucrose or in ionic media, with symmetrical or asymmetrical distributions of the ions. Moreover DEPC did not increase the K^+ permeability of the membrane (fig.4). Therefore we tentatively propose that DEPC modifies the monoamine carrier. Other reagents which inhibit ΔpH-driven monoamine uptake have been reported, but their site of action has not been characterized [19] or their effect is reversible, e.g., reserpine [20] or tetrabenazine (unpublished).

If [^{3}H]tetrabenazine binding sites are assumed to be the active site(s) of the monoamine carrier [11], the lack of effect of DEPC on K_d and B_{max} suggests that substrate binding is unaffected by the chemical modification. The absence of protection by the substrate supports this hypothesis. The observed decrease of V_{max} would then indicate that the rate or the nature of the limiting step has been affected, directly or indirectly, by DEPC-treatment. It has to be noted that electroneutral and electrogenic uptakes were similarly inhibited, suggesting a common limiting step for both processes. The determination of the nature of this step will require more information.

Acknowledgements

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